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ANALYSIS OF THE AMINO ACID AND SUGAR COMPOSITION OF STREPTOCOCCAL CELL WALLS BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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SUMMARY

A procedure for determining the amino acid and sugar composition of streptococcal peptidoglycan–polysaccharide complexes by capillary gas chromatography–mass spectrometry (GC–MS) was established. Amino acids are analysed as butyl heptafluorobutyl derivatives and sugars as alditol acetates. These two different groups of compounds are derivatized independently but chromatography in both cases utilizes the same OV-1701 fused-silica capillary column which simplifies GC–MS analysis. The butyl heptafluorobutyl procedure incorporates new pre- and post-derivatization clean-up steps. Additionally, selected-ion monitoring MS allows amino acids to be readily analysed without interference from background noise.

INTRODUCTION

The determination of the sugar and amino acid content of bacterial cell walls provides precise information on their composition and purity. As an example, the peptidoglycan component of the cell wall of the group A *Streptococcus* consists of a backbone of alternating units of muramic acid and glucosamine with attached peptide sidechains consisting of pentapeptides (L-alanine-D-glutamic acid-L-lysine-D-alanine-D-alanine) or tetrapeptides (L-alanine-D-glutamic acid-L-lysine-D-alanine). Many of these sidechains are crosslinked by L-alanine-L-alanine dipeptides[1]. The group-specific polysaccharide consists of a rhamnose backbone with glucosamine sidechains[2]. Common contaminants in cell wall preparations are proteins and membrane glycolipids. These contaminants can be readily observed by the presence of non-cell wall amino acids or sugars (*e.g.* aspartic acid or glucose respectively) and detected by selected-ion monitoring (SIM) mass spectrometry (MS). Furthermore, bacterial cell walls often contain unusual sugars and amino acid constituents (for example muramic acid, ornithine and diaminopimelic acid)[3]. The most commonly used approach for analysis of protein amino acids is the liquid chromatography-based amino acid analyser. This method is rapid, employing post-column derivatization, but has the disadvantage that if non-protein amino acids are present they are

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not easily identified. Unusual cell wall amino acids can however be identified by gas chromatography–mass spectrometry (GC–MS)[4].

We have previously described in a series of articles the development of an alditol acetate procedure for analysis of neutral and amino sugars derived from bacterial cell walls by capillary GC–MS[5–7]. Here, we describe a procedure for analysis of amino acids; the other major constituents of bacterial cell walls. Amino acids are analysed as butyl heptafluorobutyl derivatives. This procedure was modified from the method of MacKenzie and Tenaschuk[8,9] developed for determining the amino acid composition of proteins. It is demonstrated that excellent separations of alditol acetates and butyl heptafluorobutyl derivatives can be achieved on the same fused-silica capillary column (OV-1701). This greatly simplifies the combined analysis of sugars and amino acids on a single GC–MS instrument.

EXPERIMENTAL

Bacterial cell wall preparation

Streptococcus pyogenes (ATCC No. 10389) was grown overnight in 40 l of Todd Hewitt Broth (BBL Microbiology Systems, Cockeysville, MD, U.S.A.) and cells were harvested using a Millipore (Bedford, MA, U.S.A.) Pellicon cassette system. The cells (approximately 60 g wet weight in 240 ml buffer) were sonicated for 90 min (Heat Systems Ultrasonics, Long Island, NY, U.S.A.) and centrifuged for 30 min at 10 000 *g*. The supernatant was removed and recentrifuged repeatedly until essentially no pellet was seen. The pooled pellets were resonicated and centrifuged again. These steps were repeated until approximately 80–90% of the material was in the supernatant. The pooled supernatants were treated sequentially with hyaluronidase (Sigma, St. Louis, MO, U.S.A.) in 0.1 *M* phosphate–0.15 *M* sodium chloride (pH 5.3) buffer; deoxyribonuclease and ribonuclease (Calbiochem-Behring, La Jolla, CA, U.S.A.) in 0.1 *M* phosphate buffer (pH 7.2); papain in 0.1 *M* phosphate buffer (pH 7.2) containing 0.001 *M* cysteine, 0.001 *M* EDTA and 0.05% sodium azide and pepsin (Sigma) in 0.012 *M* (pH 2.0) hydrochloric acid. The amount of each enzyme was 0.0025 mg per mg wet weight of cells. The cell walls were not isolated between each step. Instead, the buffers were changed between each enzyme treatment by dialysis overnight at 4°C and the digestion itself was performed while dialysing at 37°C for 6 h. Several drops of chloroform were added to the buffer to eliminate bacterial contamination. The cell wall was collected after the final enzyme treatment and washed by repeated centrifugation at 111 000 *g* and extracted three times with choloroform–methanol–water (34:17:10) at room temperature. The water phase was then dialysed against water and lyophilized to allow storage.

Cell wall hydrolysis and derivatization for carbohydrate analysis

The cell wall fractions were analysed by the alditol acetate procedure and GC–MS which has been previously described[7]. In brief, 300 μg–1 mg (dry weight) of cell wall samples were hydrolysed in 0.5 ml of 1 *M* sulfuric acid under vacuum at 100°C for 3 h. Arabinose (5 μg) and methylglucamine (5 μg) (as internal standards for neutral and amino sugars) were added and neutralized with 40% N,N-dioctylmethylamine (Fluka, Ronkonkoma, NY, U.S.A.) in chloroform. The aqueous layer of each hydrolysis mixture was then extracted on a C_{18} column (Analytichem, Harbor

City, CA, U.S.A.). The samples were reduced with 50 μl of sodium borohydride (100 mg/ml) at 37°C for 90 min. A volume of 2 ml of acetic acid–methanol (1:200, v/v) was added to each sample which were then evaporated to near dryness at 60°C under vacuum, repeated four additional times and were allowed to dry for 3 h after the last evaporation. After cooling, 300 μl of acetic anhydride was added to each vial and the samples were heated at 100°C for 13–16 h. Water (1 ml) was added to each vial and left for 30 min. Chloroform (1 ml) was added to each, and the aqueous phases removed and discarded. To each chloroform phase, 0.8 ml of cold ammonium hydroxide (80%, v/v) was added. The mixtures were each poured onto a magnesium sulfate (Chem Elut) column (Analytichem) and eluted with 2 ml chloroform. The chloroform solutions were evaporated to dryness under vacuum and redissolved in about 40 μl of chloroform before analysis.

Cell wall hydrolysis and derivatization for amino acid analysis

Cell walls were analysed by a modified version of the butyl heptafluorobutyl procedure[8,9]. Streptococcal cell walls (300 μg dry weight) were hydrolysed in 1.0 ml 6 *M* hydrochloric acid for 6 h at 150°C under vacuum. The samples were then evaporated to dryness in a lyophilizer in a flask equiped with a sodium hydroxide trap to avoid acid contamination of the vacuum pump and refrigeration unit. The samples were then redissolved in 1.0 ml of water which were extracted on a C_{18} column (Analytichem). The samples were dried under nitrogen, 1 ml of dichloromethane was added and evaporated to dryness. The esterification reagent was prepared just prior to use. Acetyl chloride (Aldrich, Gold label) was added to *n*-butanol (Burdick and Jackson, Muskegon, WI, U.S.A., HPLC-grade), in the ratio of 1:4. A volume of 50–100 μl of this reagent was added to each vial which were gently flushed with nitrogen. Esterification was performed at 120°C for 20 min. The samples were then cooled in ice and the excess reagent removed in a stream of nitrogen. Dichloromethane (1 ml) was added and evaporated to dryness. The acylation reagent was prepared by mixing ethyl acetate (Aldrich, gold label) and heptafluorobutyric anhydride (Fluka) in a ratio of 1:1. A volume of 200 μl of the acylation reagent was added to each vial which were gently flushed with nitrogen. Acylation was performed at 150°C for 12 min. After cooling, 0.5 ml chloroform and 2 ml 1 *M* phosphate buffer was added to each. The chloroform phases were removed and evaporated to dryness. The samples were then redissolved in approximately 50 μl ethyl acetate and 1-μl samples were analyzed by GC–MS.

Chromatography of sugars and amino acids

GC–MS analyses were carried out using a 5970 mass-selective detector (Hewlett-Packard, Palo Alto, CA, U.S.A.) and a HP-5890 gas chromatograph containing a 25 m × 0.22 mm CPSil-19 (OV-1701 bonded phase) fused-silica column (Chrompack, Raritan, NJ, U.S.A.). Samples were analysed in the total ion and selected-ion mode. Tables I and II summarize retention times and ions chosen for selected-ion monitoring of specific amino acids and sugars. For amino acids generally one ion (the base peak) was selected although in some instances two prominent ions were selected. For sugars prominent high-molecular-weight fragments characteristic of particular classes of carbohydrate were selected. For analysis of alditol acetates the injection was in the splitless mode at 100°C, held for 45 s then programmed at

TABLE I

RETENTION TIMES AND IONS MONITORED IN THE ANALYSIS OF *n*-BUTYL HEPTAFLUOROBUTYL DERIVATIVES OF AMINO ACID CONSTITUENTS OF STREPTOCOCCAL CELL WALLS

I.S. = Internal standard.

Amino acid	*Ion(s) selected (m/z)*	*Time monitored (min)*
α-Aminoisobutyric acid (I.S.)	254.00	6.0–7.5
Alanine	240.00	6.0–7.5
Valine	268.00	7.5–10.0
Glycine	226.50	7.5–10.0
Threonine	253.00	7.5–10.0
Isoleucine	282.00/253.00	7.5–10.0
leucine	282.00/240.00	7.5–10.0
Serine	239.00	7.5–10.0
Norleucine (I.S.)	282.00	7.5–10.0
Proline	266.00	10.0–14.0
Cysteine	240.00	10.0–14.0
Methionine	253.00	10.0–14.0
Aspartic acid	340.00/239.00	10.0–14.0
Phenylalanine	148.00	10.0–14.0
Glutamic acid	252.00/280.00	14.0–18.0
Tyrosine	360.00	14.0–18.0
Ornithine (I.S.)	266.00	14.0–18.0
Lysine	280.00	14.0–18.0
Arginine	266.00/492.00	14.0–18.0
Tryptophan	326.00	14.0–18.0
Histidine	407.00	18.0–22.7
Cystine	284.00/340.00	18.0–22.7

30°C/min to 230°C and then at 4°C/min to 250°C and 3°C/min to 265°C and held for 4.5 min. The injector temperature was 250°C and the temperature of the interface was 270°C. For analysis of butyl heptafluorobutyrates the injection was in the splitless mode at 85°C, held for 45 s then programmed at 10°C/min to 280°C and then held

TABLE II

RETENTION TIMES AND IONS MONITORED IN THE ANALYSIS OF ALDITOL ACETATE DERIVATIVES OF CARBOHYDRATE CONSTITUENTS OF STREPTOCOCCAL CELL WALLS

Carbohydrate	*Ion selected (m/z)*	*Time monitored (min)*
Rhamnose	201.00	6.0–13.5
Arabinose (I.S.)	289.05	6.0–13.5
Glucose	289.05	6.0–13.5
Muramic acid	403.15	13.5–16.5
Glucosamine	318.00	13.5–16.5
Galactosamine	318.00	13.5–16.5
Methylglucamine (I.S.)	327.25	13.5–16.5

for 1.5 min. The injector temperature was 250°C and the temperature of the mass spectrometer interface was 290°C.

RESULTS AND DISCUSSION

Development of a method for determination of the amino acid composition of streptococcal cell walls

The butyl heptafluorobutyl procedure has predominantly been used for GC and GC–MS analysis of protein amino acids[10]. We have adapted the method for the analysis of peptidoglycan–polysaccharide complexes where a large proportion of the sample is carbohydrate. The original method produces one peak for each amino acid[8,9] and entails several processing steps prior to chromatographic analysis. These include hydrolysis in hydrochloric acid, removal of the acid followed by butyl esterification of carboxyl groups with butanol–acetyl chloride and then heptafluorobutylation of amino, hydroxyl and sulfhydryl groups with heptafluorobutyric anhydride. Long hydrolysis times (24 h at 100°C) were employed. We chose to use a shorter hydrolysis time performed at higher temperature (6 h at 150°C) which has been recently recommended[11]. We also incorporated two clean-up steps based on the use of hydrophobic C_{18} columns pre-derivatization and an aqueous phosphate buffer extraction post-derivatization[5,12]. The former step removes fatty acids and other hydrophobic materials that might be present. The latter step removes heptafluorobutyric acid generated from the acylating reagent (heptafluorobutyric anhydride), eliminates many extraneous peaks and decreases baseline drift. The presence of highly acidic reagents such as heptafluorobutyric anhydride in the analytical sample should be avoided since they can rapidly damage the stationary phase and thus greatly shorten column life.

Fig. 1 demonstrates the separation on an OV-1701 fused-silica capillary column of a mixture of the butyl heptafluorobutyl derivatives of nineteen protein amino

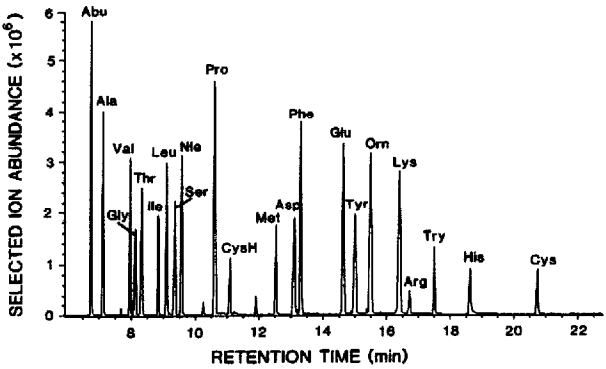

Fig. 1. Selected ion chromatogram of a mixture of butyl heptafluorobutyl derivatives separated on an OV-1701 fused-silica capillary column. Abu = α-aminoisobutyric acid (internal standard), Ala = alanine, Val = valine, Gly = glycine, Thr = threonine, Ile = isoleucine, Leu = leucine, Ser = serine, Nle = norleucine (internal standard), Pro = proline, CysH = cysteine, Met = methionine, Asp = aspartic acid, Phe = phenylalanine, Glu = glutamic acid, Tyr = tyrosine, Orn = ornithine (internal stanard), Lys = lysine, Arg = arginine, Try = tryptophan, His = histidine, Cys = cystine.

acids (30 μg of each taken through the entire procedure). These were alanine, valine, glycine, threonine, leucine, isoleucine, serine, proline, cysteine, methionine, aspartic acid, phenylalanine, glutamic acid, tyrosine, lysine, arginine, tryptophan, histidine and cystine and internal standards (α-aminoisobutyric acid, norleucine, and ornithine). α-Aminoisobutyric acid was used as an internal standard for alanine; norleucine was used for valine through phenylalanine; ornithine for glutamic acid through histidine. Three internal standards were selected as it was found that better reproducibility was obtained if the standard eluted close to the peak of interest. These internal standards do not often occur in proteins or cell walls. Although ornithine is occasionally found in certain bacterial cell walls it is not present in group A streptococci. All peaks were base-line resolved. During derivatization glutamine is converted to glutamic acid and asparagine to aspartic acid and thus each pair of amino acids produce one chromatographic peak[10]. Certain amino acids possessing nitrogen-containing moieties are known to be difficult to derivatize; arginine contains a guanidino group and histidine an imidazole group in the sidechain[9]. Arginine was found to produce a small peak relative to other amino acids. Arginine requires vigorous acylation conditions and the absence of water. In the presence of even small amounts of water heptafluorobutyric acid is formed which has been noted to considerably reduce the yield of the heptafluorobutyl derivative of arginine[13]. We further note that in addition to problems in the formation of arginine derivatives this peak disappears rapidly on storage in ethyl acetate. As noted previously histidine produces two peaks which represent the mono- and diacyl derivatives. In the case of the diacyl derivative the imidazole nitrogen is heptafluorobutylated. The second peak tails, a property noted by McKenzie and Tenaschuk[9] of the monoacyl derivative. In the selected ion chromatogram (Fig. 1) only the second histidine peak is seen since the ion monitored, *m/z* 407 (the molecular ion for butyl monoacyl histidine), is considerably less abundant in the first histidine peak.

The major components of streptococcal cell walls are amino acids and carbohydrates. Sugars contain hydroxyl (and sometimes amino) groups and sugar-derived peaks can be produced from reaction with heptafluorobutyl anhydride. When glucose is mixed with free amino acids and butyl heptafluorobutyl derivatives prepared, carbohydrate-derived peaks are produced that co-elute with derivatives of

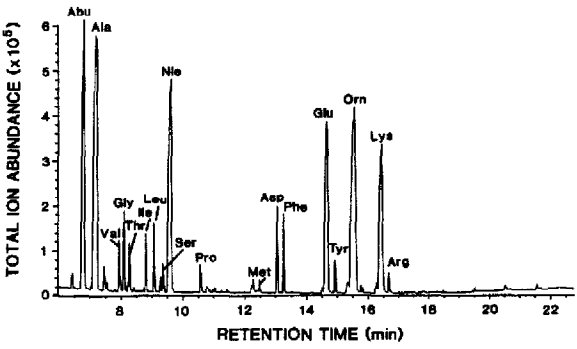

Fig. 2. Total ion chromatograms of butyl heptafluorobutyl derivatives of a hydrolysate of a streptococcal cell wall preparation separated on an OV-1701 fused-silica capillary column. Peak identification as in Fig. 1.

hydroxyproline, proline, methionine, and aspartic acid[14]. It was important to establish experimentally in the present study whether carbohydrate-derived peaks interfered in the analysis of streptococcal cell walls. Fig. 2 shows a total ion chromatogram of butyl heptafluorobutyl derivatives of a hydrolysate of a streptococcal cell wall preparation separated on an OV-1701 fused-silica column. The major peaks in this

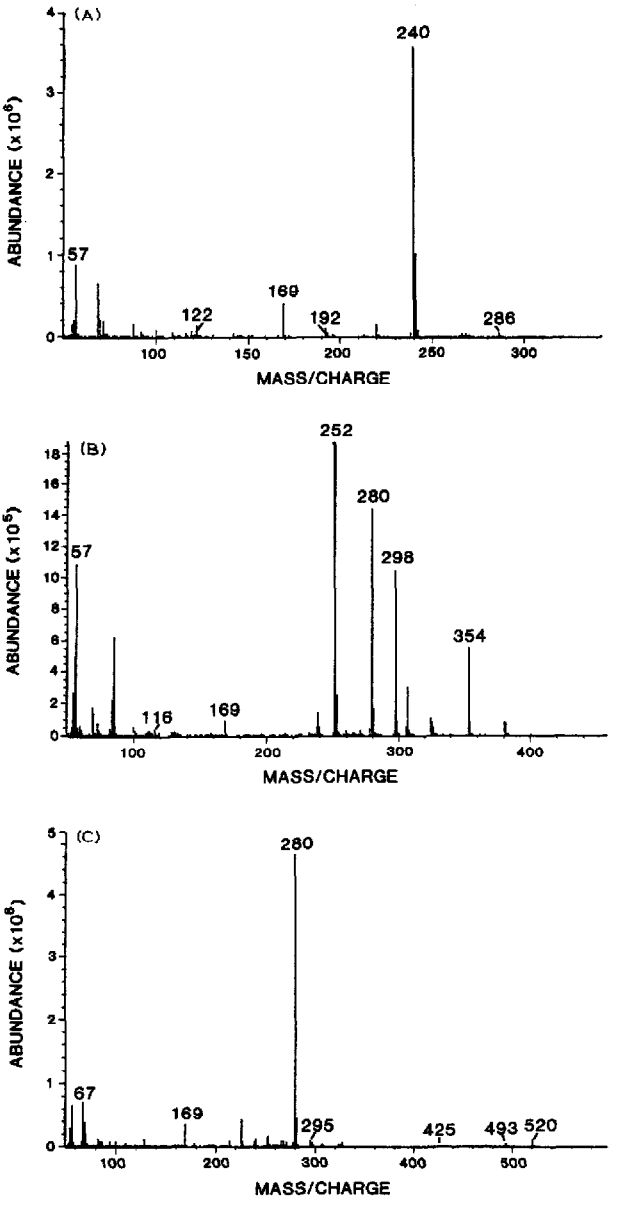

Fig. 3. Electron ionization mass spectra (70 eV) of amino acids isolated from group A streptococcal cell walls and analysed as butyl heptafluorobutyl derivatives. (A) Alanine, (B) glutamic acid, (C) lysine.

chromatogram other than internal standards are alanine, lysine and glutamic acid as expected. The mass spectra of these three amino acids are given in Fig. 3. We did not find any major peak that could have been carbohydrate-derived. Examination of the mass spectra of minor peaks established that the majority were derived primarily from protein amino acids and only a few remained unidentified and could be carbohydrate-derived. Extensive heating of sugars in hydrochloric acid has been demonstrated to destroy them and this may explain their absence in chromatograms of cell wall hydrolysates[15]. Tryptophan was not observed in any cell wall chromatogram since it does not survive acid-catalysed hydrolysis[10].

Using SIM for major ions present at the correct retention time for all amino acids of interest it was possible to simplify chromatograms. Butyl heptafluorobutyl derivatives generally produce an abundant ion between m/z 200 and m/z 300 derived from the parent structure. The use of these high-mass ions for SIM provides excellent sensitivity and selectivity. Background signal is primarily produced by fragmentation of the heptafluorobutyl group (mainly ions m/z 119 and 147). Fig. 4 shows a selected ion chromatogram of butyl heptafluorobutyl derivatives of a hydrolysate of a streptococcal cell wall preparation separated in an OV-1701 fused-silica capillary column. The major peaks (alanine, lysine and glutamic acid) are readily visualized in both total and selected ion modes. However, chromatograms are simplified in the SIM mode by removal of a number of unidentified minor peaks. Thus contaminating protein amino acids can be more confidently observed.

The same OV-1701 column used for separation of amino acid mixtures was used for analysis of carbohydrates. Fig. 5 illustrates the separation of a mixture of alditol acetates from a purified streptococcal cell wall hydrolysate; before derivatization the free sugars were generated by heating in 1 *M* sulfuric acid for 3 h at 100°C. The major peaks are rhamnose, muramic acid and glucosamine. A small amount of glucose can be seen. This may be derived from contaminating membrane glycolipid[16,17]. However, it has been suggested that certain group A streptococcal cell walls contain a covalently bound glucose containing polysaccharide (polysaccharide G)[1]. The source of glucose is thus unclear at this time.

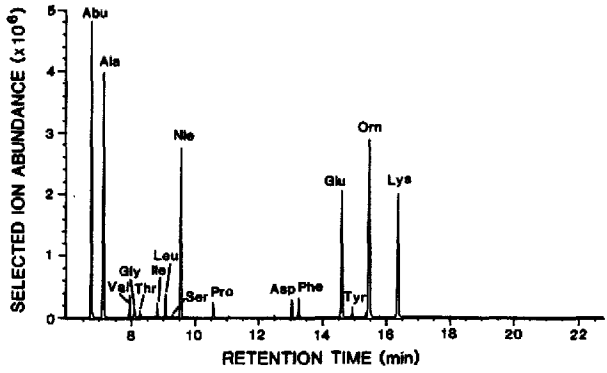

Fig. 4. Selected ion chromatogram of butyl heptafluorobutyl derivatives of a hydrolysate of a streptococcal cell wall preparation separated on an OV-1701 fused-silica capillary column. Peak identification as in Fig.

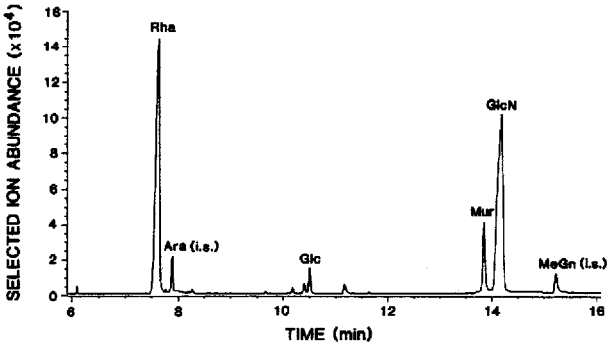

Fig. 5. Single ion chromatogram of alditol acetate derivatives of a hydrolysate of a streptococcal cell wall preparation separated on an OV-1701 fused-silica capillary column. Peak identification: Rha = rhamnose, Ara = arabinose (internal standard), Glc = glucose, Mur = muramic acid, GlcN = glucosamine, and MeGn = methylglucamine (internal standard).

TABLE III

THE SUGAR AND AMINO ACID COMPOSITION OF THREE GROUP A STREPTOCOCCAL CELL WALL PREPARATIONS

Each component is expressed as % of the dry weight of the sample.

Compound	*Cell wall preparation*		
	A	*B*	*C*
Amino acid			
Alanine	13.4	9.2	12.5
Valine	0.4	1.2	0.9
Glycine	0.6	1.3	1.3
Threonine	0.2	0.5	0.2
Isoleucine	0.4	1.1	0.7
Leucine	0.5	1.2	0.9
Serine	0.2	0.3	0.1
Proline	0.4	1.2	0.7
Cysteine	0.1	0.1	–
Methionine	–	–	–
Aspartic acid	1.4	3.4	2.4
Phenylalanine	0.3	0.8	0.3
Glutamic acid	7.7	6.2	6.2
Tyrosine	0.4	1.5	0.4
Lysine	7.5	6.7	6.4
Arginine	0.8	0.9	0.1
Tryptophan	–	–	–
Histidine	–	–	–
Cystine	–	–	–
Carbohydrate			
Rhamnose	25.4	23.4	22.8
Glucose	1.0	2.2	1.2
Muramic acid	8.1	7.8	7.5
Glucosamine	21.5	22.6	16.9

Application of the method to routine characterization of cell walls

The purification of group A streptococcal and other Gram-positive cell walls (peptidoglycan–polysaccharide complexes) is often a tedious procedure. The isolation of bacterial cell walls generally involves an initial step in which the cell is mechanically broken. This cellular breakage releases intracellular constituents and allows access to solvents and enzymes during the purification process. The unbroken cells are removed from the cell wall preparation by low speed centrifugation. The crude cell walls are then treated in a stepwise fashion with multiple enzymes and solvents to remove unwanted cellular constituents including nucleic acids, proteins and lipids. At each purification stage the walls are usually separated from released contaminants with repeated pelleting and washing with aqueous buffer[17].

We developed an alternative appraoch in which streptococcal cell wall particles can be simply prepared by enzymatic treatment of supernatants of sonicated cells without the need for numerous centrifugation and washing steps. The enzymatic treatments are now performed sequentially in dialysis bags. Products of enzymatic treatment are removed continuously by dialysis. To maintain optimum efficiency of the five enzymes used (hyaluronidase, ribonuclease, deoxyribonuclease, papain and pepsin) reaction buffers are also changed by dialysis. Enzymes used in the preparation and other residual proteins or peptides derived from the streptococci are removed by the final ultracentrifugation steps.

Table III summarizes the composition of three separate batches of streptococcal cell walls. In each instance the major sugars are rhamnose, muramic acid and glucosamine and the major amino acids are alanine, glutamic acid and lysine. These constituents account for in excess of 80% of the dry weight of these preparations. A further 10% is accounted for by the minor protein amino acids and glucose. The purity of these preparations are comparable to those prepared previously even though the purification procedure described here is considerably easier to perform[17]. The molar ratios for lysine–glutamic–alanine–muramic acid in the three preparations were: preparation A, 1.0:1.0:2.9:0.6; preparation B, 1.0:0.9:2.2:0.7; preparation C, 1.0:1.0:3.2:0.7. The muramic acid values are slightly lower than would be expected, however the overall results agree with classic studies on the structure of streptococcal peptidoglycan–polysaccharide complexes.

CONCLUSION

The determination of the carbohydrate and amino acid composition of streptococcal peptidoglycan–polysaccharide preparations can be readily accomplished by GC–MS. Sugar components (rhamose, muramic acid and glucosamine) are analysed as alditol acetates. Amino acid components (alanine, glutamic acid and lysine) are analysed as butyl heptafluorobutyrates. The use of pre-derivatization clean-up with hydrophobic columns and post-derivatization clean-up with aqueous extraction provides selective and rapid clean-up for amino acids in comparison to the common use of ion-exchange resins pre-derivatization. Additionally SIM-MS simplified chromatograms. Both alditol acetates and butyl heptafluorobutyrates can be separated on the same OV-1701 fused-silica capillary column. The major cell wall components and many minor non-cell wall contaminants are readily identified by MS. The ability to perform both sets of analyses on one GC column is important since it allows the

routine use of the GC–MS instrument for sugar and amino acid analyses without the need to switch columns. The combined GC–MS analysis of sugars and amino acids has been rare. The approach described here may have wide aplication to the analysis of macromolecules that contain both carbohydrate and amino acid constituents.

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